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Biochemistry

Bovine pancreatic trypsin inhibitor–trypsin complex as a detection system for recombinant proteins

(protein tagging/human growth hormone/rhodopsin)

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ABSTRACT **Bovine pancreatic trypsin inhibitor (BPTI) binds to trypsin and anhydrotrypsin (an enzymatically inactive derivative of trypsin) with affinities of 6×10^{-14} and 1.1×10^{-13} M, respectively. We have taken advantage of the high affinity and specificity of this binding reaction to develop a protein tagging system in which biotinylated trypsin or biotinylated anhydrotrypsin is used as the reagent to detect recombinant fusion proteins into which BPTI has been inserted. Two proteins, opsin and growth hormone, were used as targets for insertional mutagenesis with BPTI. In each case, both domains of the fusion protein appear to be correctly folded. The fusion proteins can be specifically and efficiently detected by biotinylated trypsin or biotinylated anhydrotrypsin, as demonstrated by staining of transfected cells, protein blotting, affinity purification, and a mobility shift assay in SDS/polyacrylamide gels.**

Most conventional protein detection systems rely on the specificity of antigen–antibody interactions. One application of this specificity is in the detection of a recombinant protein by engineering the addition of a small peptide epitope, a method referred to as protein tagging (1). This approach has been used for various purposes, including immunoaffinity purification and determination of subcellular localization. In this paper we describe an alternative protein detection system based upon insertion of bovine pancreatic trypsin inhibitor (BPTI) into a recombinant protein followed by binding of the hybrid protein to trypsin or a trypsin derivative. As described below, the high affinity and specificity of the BPTI–trypsin complex make this an attractive system for a number of applications.

BPTI is a 58-amino acid protein that binds with high affinity to trypsin and other serine proteases. The BPTI–trypsin complex is extremely stable, with a dissociation constant of 6×10^{-14} M and a half-life of ≈17 weeks at pH 8.0, 25°C (2). A similar complex formed between BPTI and anhydrotrypsin, an enzymatically inactive derivative of trypsin, has a dissociation constant of 1.1×10^{-13} M and a half-life of ≈13 weeks at pH 8.0, 25°C (3). At room temperature, the BPTI–trypsin complex is resistant to dissociation in 8 M urea or 6 M guanidine hydrochloride (4, 5). The crystal structure of the BPTI–trypsin complex shows that the N and C termini of BPTI are within 8 Å of each other, but >20 Å from the site of trypsin binding (Fig. 1; ref. 6).

A BPTI–trypsin detection system could be superior to an epitope–monoclonal antibody (mAb) detection system in several respects. The close proximity of the N and C termini of BPTI suggests that its insertion into surface loops would minimally perturb the target protein while still allowing efficient binding to trypsin; the stability of the BPTI–trypsin complex should allow the BPTI-tagged protein to be assayed under extreme pH, temperature, and solvent conditions that would disrupt typical antigen–antibody interactions; and the availability of pure trypsin at a modest cost should make large-scale experiments relatively inexpensive.

In the present study, we describe the characteristics of the BPTI–trypsin system using bovine opsin and human growth hormone (hGH) as target proteins for insertional mutagenesis.

MATERIALS AND METHODS

DNA and Cells. The mammalian expression vector pCIS (7), bovine opsin cDNA (8), rabbit GH receptor cDNA (9), hGH gene (10), and BPTI coding region (11) have been described. For BPTI insertion, a unique *Bam*HI site was engineered into the bovine opsin cDNA by replacing codons 194 to 195 with GGATCC (encoding Gly and Ser). The hGH gene was similarly modified at codons 143 to 144 or 149 to 150 or by insertion of the *Bam*HI site after codon 191. The BPTI coding region was PCR amplified using primers flanked by *Bam*HI sites. The amplified BPTI was cloned into the unique *Bam*HI site of the target DNA. All constructs were expressed by transient transfection of human embryonic kidney cells (293S) using the pCIS vector (7).

Preparation and Biotinylation of Anhydrotrypsin. Anhydrotrypsin was prepared as described (12). Two hundred milligrams of trypsin (Worthington) was dissolved in 100 ml of phosphate-buffered saline (PBS) and allowed to react with 37 mg of phenylmethylsulfonyl fluoride (PMSF; dissolved in 1 ml of acetone) for 30 min at 25°C. The reaction was terminated by the addition of 1 M HCl to lower the pH to ≈3, and the sample was dialyzed against 1 mM HCl overnight at 4°C to remove free PMSF. Base elimination of PMS from PMS–trypsin was achieved by incubation with 1/19 volume of 1 M KOH for 10 min on ice. The sample was adjusted to 1 mg of protein per ml and 50 mM sodium borate (pH 8.6) and stored at −80°C. The anhydrotrypsin preparation had <2% of the enzymatic activity of native trypsin as determined using the chromogenic substrate N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPNA; Sigma). To inhibit any residual proteolytic activity, the buffers used with anhydrotrypsin contained 0.2 mM PMSF. Biotinylation of trypsin and anhydrotrypsin was carried out with biotin-C_{11}-*N*-hydroxysuccinimide ester followed by removal of free biotinylation reagent on a Sephadex G-25 column.

Spectroscopic Assay of Opsin and Opsin–BPTI. Two days after transfection the cells were harvested, and total cell membranes were purified on a sucrose step gradient, detergent solubilized, and reconstituted with 11-*cis*-retinal (13).

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; hGH, human growth hormone; mAb, monoclonal antibody; BAPNA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; HRP, horseradish peroxidase.

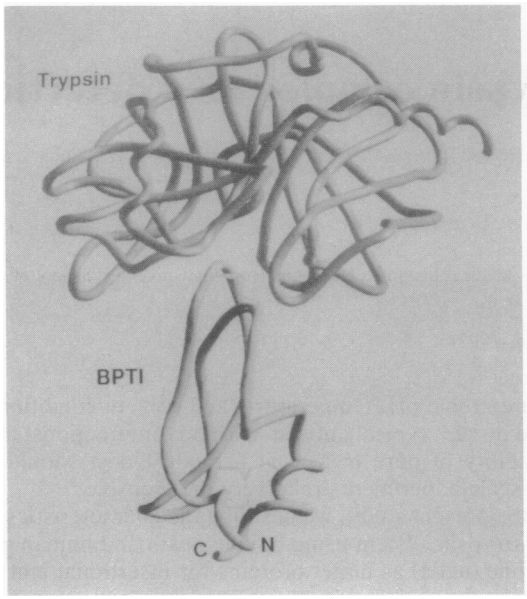

FIG. 1. Structure of the complex between bovine trypsin and BPTI (6).

Photobleaching difference spectra were recorded in the presence of hydroxylamine as described (13).

Preparation of hGH and hGH–BPTI. To prepare nonradiolabeled hGH and hGH–BPTI, transfected cells were placed in serum-free Dulbecco's modified Eagle's medium (DMEM) 18 hr after transfection and the medium was harvested 48 hr later. To prepare radiolabeled hGH and hGH–BPTI, transfected cells were labeled with [^{35}S]methionine for 30 min in serum-free DMEM and incubated for an additional 2 hr in serum-free DMEM prior to harvesting and concentrating the medium.

Affinity Purification. For affinity purification of hGH and hGH–BPTI, total cell protein was labeled with [^{35}S]methionine for 30 min in serum-free DMEM, and a cell lysate was prepared in 0.5 ml of 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, and 0.2 mM PMSF per 10-cm plate. Fifty percent of the lysate from one 10-cm plate was precleared with avidin–agarose beads (Pierce), incubated with 20 pmol of biotinylated anhydrotrypsin for 30 min at 37°C, and then further incubated with gentle agitation for 1 hr at 4°C with 10 μl of a 50% slurry of avidin–agarose beads. The avidin–agarose beads were recovered by centrifugation, washed five times with lysis buffer, mixed with Laemmli buffer, and heated to 85°C for 5 min prior to SDS/PAGE.

Protein Blots. Recombinant proteins were detected using the enhanced chemiluminescence (ECL) method (Amersham) with appropriate antibody probes: mouse mAb 1D4 for opsin (14) and polyclonal rabbit antibody (BioDesign, New York) for hGH. To detect BPTI fusion proteins with biotinylated trypsin or biotinylated anhydrotrypsin, blots were pretreated with 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.2% Tween (TBST) containing 5% dry milk for 30 min, incubated in TBST with 1 μg of biotinylated trypsin or biotinylated anhydrotrypsin per ml for 30 min, washed five times over 30 min with TBST, and incubated with horseradish peroxidase (HRP) conjugated to avidin D (Vector Laboratories) at a dilution of 0.1 μg/ml followed by ECL detection. All procedures were carried out at room temperature. Blots were reprobed after overnight drying at room temperature, a treatment that inactivates HRP.

Mobility Shift Assay. For opsin–BPTI, 0.5% of the Chaps-solubilized membrane sample used for absorption spectroscopy (corresponding to 10% of the material derived from one 10-cm plate) or for hGH–BPTI, 0.3% of the conditioned medium from one 10-cm plate was incubated with the indicated quantity of biotinylated anhydrotrypsin for 30 min at 37°C. For prebinding experiments, BPTI (Worthington) or bovine serum albumin (BSA) was incubated with biotinylated anhydrotrypsin for 30 min at 37°C prior to incubation with BPTI fusion proteins. The samples were then mixed with an equal volume of Laemmli buffer without reducing agent and loaded at room temperature onto an SDS/12% polyacrylamide gel. Biotinylated anhydrotrypsin was visualized by blotting the gel and probing with avidin–HRP as described above.

Cell Staining to Detect BPTI Fusion Proteins. 293S cells were grown on untreated glass coverslips and fixed and permeabilized 1 day after transfection with 90% methanol. To detect BPTI fusion proteins, coverslips were incubated with 1 μg of biotinylated trypsin or biotinylated anhydrotrypsin per ml in PBS for 30 min, washed several times in PBS, incubated with preformed avidin–biotin–HRP complex (Vector Laboratories), washed several times in PBS, and allowed to react with diaminobenzidene. All procedures were carried out at room temperature. Cells were stained with mAb 1D4 to bovine opsin or a mAb to hGH (no. 901; BioDesign). Stained cells were mounted in 50% glycerol/PBS. To detect expressed rabbit GH receptor using hGH–BPTI, transfected cells were grown for 1 day as described above, washed twice with cold PBS containing 0.02% sodium azide, and incubated with recombinant hGH–BPTI (conditioned medium from transfected 293S cells diluted 1:10) for 1 hr at room temperature. After several washes with PBS, the cells were methanol fixed and processed as described above for detection of BPTI fusion proteins. For prebinding experiments with hGH, cells were first incubated with hGH (undiluted conditioned medium from transfected 293S cells), washed three times in PBS, and then processed as described for detection by hGH–BPTI binding.

RESULTS

Preparation and Characterization of Anhydrotrypsin. Anhydrotrypsin is an enzymatically inactive form of trypsin in which the active site Ser-195 has been chemically converted to dehydroalanine. Anhydrotrypsin was prepared by PMSF treatment followed by base elimination of PMS from the PMS–trypsin complex as described (12). The binding capacity of anhydrotrypsin for BPTI was determined by a quantitative BPTI inhibition assay (3) and a mobility shift assay of the complex between BPTI and anhydrotrypsin in SDS/polyacrylamide gels.

In the inhibition assay, increasing quantities of BPTI were incubated with 0.3 μM anhydrotrypsin at 25°C, pH 8.0. After 40 min, trypsin was added to 0.3 μM and allowed to bind the remaining free BPTI during an additional 10-min incubation. Trypsin activity was then measured spectrophotometrically with BAPNA as a chromogenic substrate. As shown in Fig. 2*A*, 0.2 μM BPTI was required to saturate the anhydrotrypsin, indicating that ≈65% of the anhydrotrypsin molecules retain BPTI binding capacity.

In the mobility shift assay, performed with biotinylated anhydrotrypsin, 0.8 nmol of biotinylated anhydrotrypsin was incubated with increasing amounts of BPTI at 37°C for 30 min (Fig. 2*B*). The samples were loaded onto an SDS/polyacrylamide gel under nondenaturing conditions, electrophoresed, and stained with Coomassie blue. As seen in Fig. 2*B*, the BPTI/biotinylated anhydrotrypsin complex resists dissociation during SDS/PAGE and has a mobility that corresponds to an apparent molecular weight significantly greater than the sum of the two component molecular weights. In this experiment, unbound BPTI was observed only at concentrations >0.6 nmol, indicating that of the original 0.8 nmol of biotinylated anhydrotrypsin, between 0.5 and 0.6 nmol can bind BPTI.

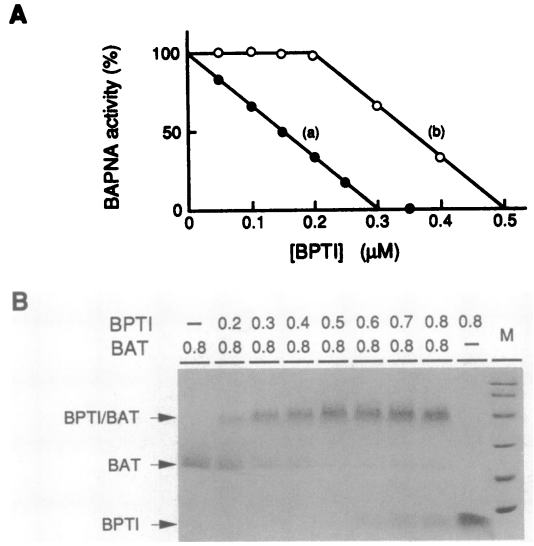

FIG. 2. Binding of anhydrotrypsin to BPTI. (*A*) Competitive binding assay. ● (a), Inhibition of trypsin (0.3 μM) by different concentrations of BPTI; ○ (b), as in (a) except BPTI was first incubated with 0.3 μM anhydrotrypsin prior to addition of trypsin. (*B*) Mobility shift assay. The quantities of the components in each sample are listed at the top: 0.8 nmol of biotinylated anhydrotrypsin (BAT) was incubated with the indicated amount of BPTI (in nmol) for 30 min at 37°C in a reaction volume of 25 μl, prior to electrophoresis on a nonreducing SDS/polyacrylamide gel and Coomassie staining. Size markers (M) are 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa (top to bottom).

Insertion of BPTI into Rhodopsin. Opsin, the apoprotein of rhodopsin, is an integral membrane protein containing seven transmembrane segments. As an initial test of the BPTI–trypsin system, a bovine opsin derivative was constructed in which BPTI was inserted into opsin's putative second extracellular loop between codons 194 and 195 (opsin–BPTI). This location was chosen because it is hydrophilic and therefore likely to be on the surface.

Opsin and opsin–BPTI were produced in 293S cells following transient transfection. The cells were fixed and permeabilized with methanol and stained with either mAb 1D4 (directed against opsin's C terminus) or biotinylated trypsin (Fig. 3). Biotinylated trypsin stained only those cells expressing opsin–BPTI, whereas mAb 1D4 stained cells expressing opsin and opsin–BPTI. Opsin–BPTI and opsin appear to accumulate primarily in the plasma membrane. With cells expressing opsin–BPTI, mAb 1D4 and biotinylated trypsin produced staining patterns that were indistinguishable.

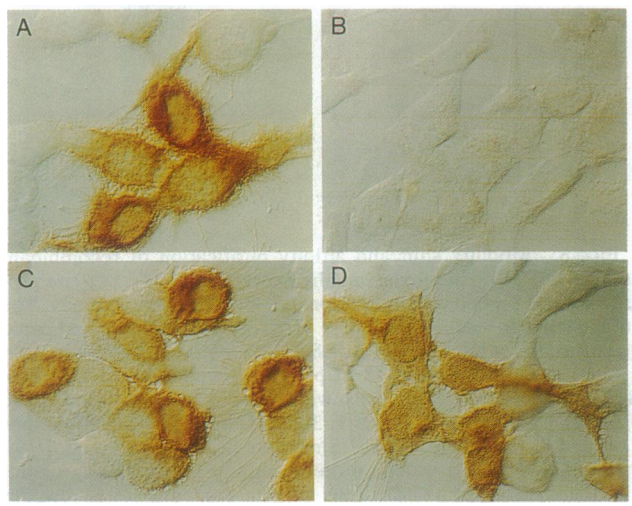

FIG. 3. Nomarski image of 293S cells transfected with an opsin expression plasmid (*A* and *B*) or an opsin–BPTI expression plasmid (*C* and *D*) and stained with anti-opsin mAb 1D4 (*A* and *C*) or biotinylated trypsin (*B* and *D*). (×550.)

As a second measure of whether the BPTI domain of the opsin–BPTI fusion protein was correctly folded, we examined the ability of biotinylated anhydrotrypsin to form a complex with opsin–BPTI by the electrophoretic mobility shift assay (Fig. 4). Membrane protein samples containing either opsin or opsin–BPTI and corresponding to 0.5% of the material used for spectral analysis were incubated with biotinylated anhydrotrypsin. In these membrane samples, opsin and opsin–BPTI constitute <1% of the protein. Increasing amounts of free BPTI were preincubated with biotinylated anhydrotrypsin to assess the specificity of the binding reaction between biotinylated anhydrotrypsin and opsin–BPTI. Following SDS/PAGE under nonreducing conditions, the gel was electroblotted and allowed to react sequentially with mAb 1D4 (Fig. 4 *Left*) to reveal opsin and opsin–BPTI and with avidin–peroxidase (Fig. 4 *Right*) to reveal biotinylated anhydrotrypsin. As seen in Fig. 4, biotinylated anhydrotrypsin is able to bind to all of the opsin–BPTI molecules to form a complex with a mobility lower than that of free opsin–BPTI. The complex is not formed with opsin, and it is blocked by preincubation with free BPTI. The right panel of Fig. 4 shows that an increase in BPTI produces a proportional increase in the fraction of biotinylated anhydrotrypsin bound to BPTI and a corresponding decrease in the fraction of biotinylated anhydrotrypsin bound to opsin–BPTI. The opsin–BPTI complex with biotinylated anhydrotrypsin can also be precipitated with avidin–agarose as described below for a hGH–BPTI fusion protein (data not shown).

In a third experiment to test the proper folding of the BPTI domain in the fusion protein, we determined whether opsin–BPTI could be specifically recognized by trypsin on a protein blot. In this experiment the membrane preparation used for spectral analysis was electrophoresed on an SDS/polyacrylamide gel, transferred to nitrocellulose, and probed with mAb 1D4 (Fig. 5 *Left*) and subsequently with biotinylated trypsin (Fig. 5 *Right*). Increasing amounts of 2-mercaptoethanol were added to the samples prior to electrophoresis to assess the requirement for disulfide bonds in the recognition of BPTI by trypsin. As seen in Fig. 5, biotinylated trypsin recognizes opsin–BPTI only under nonreducing conditions.

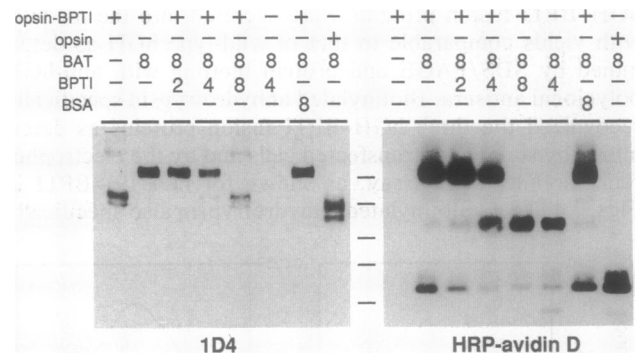

FIG. 4. Mobility shift assay of the binding of biotinylated anhydrotrypsin (BAT) to opsin–BPTI in a nonreducing SDS/polyacrylamide gel. The protein blot was first probed with anti-opsin mAb 1D4 (*Left*) and then reprobed with avidin–HRP to visualize biotinylated anhydrotrypsin (*Right*). The components in each sample are listed at the top: "+" indicates 0.5% of the opsin or opsin–BPTI sample used for spectral analysis (Fig. 6); the indicated amounts (pmol) of BPTI and BSA were preincubated with biotinylated anhydrotrypsin prior to addition of opsin or opsin–BPTI. Horizontal bars indicate protein size standards of 80, 49.5, 32.5, and 27.5 kDa (top to bottom).

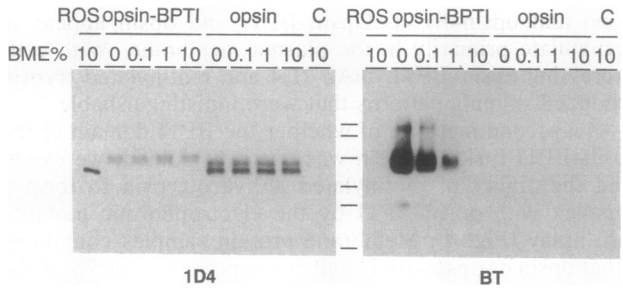

FIG. 5. Protein blot showing the effect of 2-mercaptoethanol (β-mercaptoethanol, BME) on the binding of opsin–BPTI to biotinylated trypsin (BT). The protein blot was first probed with anti-opsin mAb 1D4 (*Left*) and then reprobed with biotinylated trypsin to visualize BPTI (*Right*). The percent BME in each sample is indicated. Bovine rod outer segments (ROS) and mock transfected 293S cell membranes (C) serve as controls. Horizontal bars indicate protein size standards of 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa.

To determine whether the opsin domain of the opsin–BPTI fusion protein was correctly folded, we examined its ability to form a photolabile pigment upon binding to 11-*cis*-retinal. Following transient transfection, detergent-solubilized cell membrane preparations containing either opsin or opsin–BPTI were incubated with 11-*cis*-retinal and the photobleaching difference spectra were measured (Fig. 6). Both proteins form photolabile pigments, the absorption spectra of which are indistinguishable from that of rhodopsin from cattle retinas. However, in three independent experiments, the yield of reconstituted opsin–BPTI was reproducibly 5- to 10-fold lower than that of opsin, consistent with the lower yield observed in protein blots (Fig. 5).

Insertion of BPTI into hGH. As a second target protein to test the BPTI–trypsin system we chose hGH, a monomeric hormone of 191 amino acids. The recently determined co-crystal structure of hGH bound to its receptor (hGHr; ref. 15) reveals that hGH, like porcine growth hormone, consists of a four-helix bundle with two disulfide bonds. Three hGH–BPTI fusion proteins were constructed in which BPTI was inserted after codons 142, 148, and 191, referred to as hGH142–BPTI, hGH148–BPTI, and hGH191–BPTI. These insertion points were chosen because in the hGH–hGHr cocrystal structure they are on the surface of hGH away from those regions that contact the receptor.

Following transient transfection of 293S cells, the three hGH–BPTI fusion proteins were secreted into the medium with yields comparable to that of wild-type hGH as determined by SDS/PAGE and protein blotting with anti-hGH polyclonal antisera. Biotinylated anhydrotrypsin specifically recognized the three hGH–BPTI fusion proteins as determined by staining of transfected cells and by the electrophoretic mobility shift assay, as shown for hGH191–BPTI in Figs. 7 and 8*A*. Biotinylated anhydrotrypsin also specifically

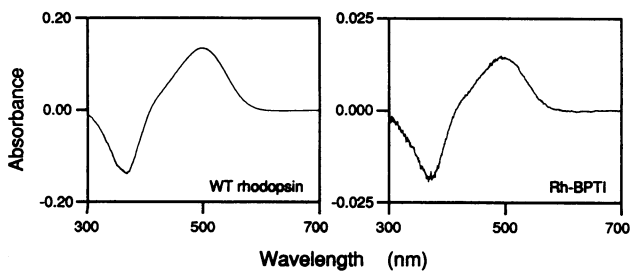

FIG. 6. Photobleaching difference absorption spectra of wild-type rhodopsin (*Left*) and rhodopsin-BPTI (*Right*) produced by transfection of 293S cells and reconstitution with 11-*cis*-retinal.

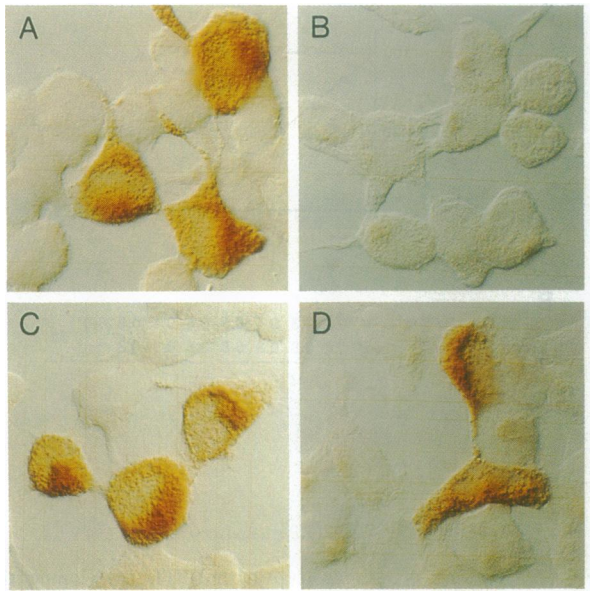

FIG. 7. Nomarski image of 293S cells transfected with an hGH expression plasmid (*A* and *B*) or an hGH191–BPTI expression plasmid (*C* and *D*) and stained with anti-hGH polyclonal antibodies (*A* and *C*) or biotinylated trypsin (*B* and *D*). (×690.)

recognized the three hGH–BPTI fusion proteins in protein blotting experiments (data not shown).

To examine the utility of the BPTI–trypsin system for affinity purification of tagged proteins, biosynthetically labeled hGH191–BPTI was purified using a protocol similar to those typically used for immunoprecipitation. Cytosolic extracts were prepared from [^{35}S]methionine-labeled cells that had been transfected with plasmids encoding either hGH or hGH191–BPTI, the extracts were incubated with biotinylated anhydrotrypsin, and then biotinylated anhydrotrypsin together with any proteins bound to it were recovered using

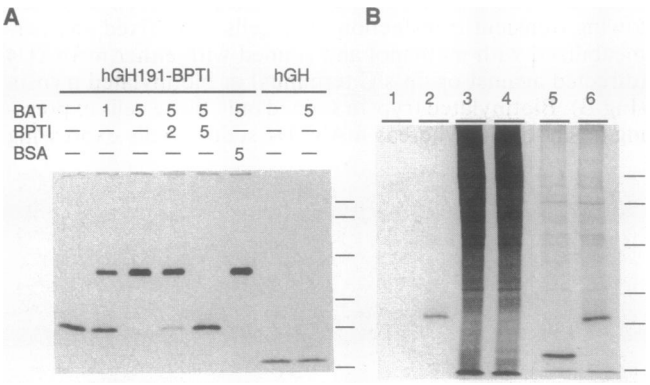

FIG. 8. Mobility shift and affinity purification of recombinant hGH191–BPTI by biotinylated anhydrotrypsin (BAT). (*A*) The protein blot was probed with a polyclonal rabbit anti-hGH antibody. The components in each sample are listed at the top: the indicated quantities (in pmol) of BPTI or BSA were preincubated with biotinylated anhydrotrypsin in 15 μl prior to the addition of hGH191–BPTI or hGH. (*B*) 293S cells were transfected with expression plasmids encoding hGH (lanes 1, 3, and 5) or hGH191–BPTI (lanes 2, 4, and 6) and labeled with [^{35}S]methionine. Lanes 1 and 2, [^{35}S]-methionine-labeled cytosolic proteins affinity purified using biotinylated anhydrotrypsin (50% of the material from one 10-cm dish per lane); lanes 3 and 4, [^{35}S]methionine-labeled cytosolic proteins (1% of the material from one 10-cm dish per lane); lanes 5 and 6, serum-free conditioned medium from [^{35}S]methionine-labeled cells (5% of the material from one 10-cm dish per lane). Protein size standards are as in Fig. 5.

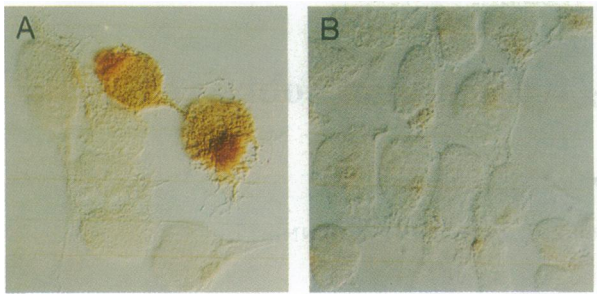

FIG. 9. Binding of hGH191–BPTI to rabbit GH receptor assayed by cell staining. (*A*) 293S cells transfected with rabbit GH receptor were incubated with hGH191–BPTI, methanol fixed, and stained with biotinylated anhydrotrypsin followed by ABC peroxidase. (*B*) As in *A* except that cells were incubated with hGH prior to incubation with hGH191–BPTI. (×690.)

avidin–agarose beads. As seen in Fig. 8*B*, hGH191–BPTI was purified to near homogeneity by this procedure.

The experiments described above show that in each of the three hGH–BPTI fusion proteins the BPTI domain is correctly folded. To determine whether the hGH domain in each fusion protein was also correctly folded, each hGH–BPTI fusion was assayed for its ability to bind recombinant rabbit GH receptor. In this experiment, cells transfected with a plasmid encoding the rabbit GH receptor were incubated with either hGH or hGH–BPTI and then stained with biotinylated anhydrotrypsin. Transfected cells incubated with hGH were not stained by biotinylated anhydrotrypsin. However, transfected cells incubated with each of the three hGH–BPTI fusion proteins were stained by biotinylated anhydrotrypsin, and this staining was abolished if the cells were preincubated with hGH prior to incubation with the hGH–BPTI fusions (Fig. 9).

DISCUSSION

This paper describes the use of BPTI as a tag for recombinant proteins. In the examples described here, opsin–BPTI and hGH–BPTI fusion proteins were shown to be efficiently and specifically recognized by anhydrotrypsin. Binding of biotinylated anhydrotrypsin to the fusion proteins could be detected by staining of fixed and permeabilized cells, by protein blotting, by an SDS/PAGE mobility shift assay, and by absorption to agarose beads. In each case the BPTI fusion protein–biotinylated anhydrotrypsin complex was detected using avidin recognition of biotinylated anhydrotrypsin. For each of these detection methods, the sensitivity of the BPTI–trypsin system compares favorably to that obtained using antibody binding. For example, in protein blotting experiments using the opsin–BPTI fusion protein, biotinylated anhydrotrypsin can routinely detect 100 pg of fusion protein.

In the fusion proteins described here, the BPTI domain is located outside of the cell, as it is *in vivo*. Within the limits of the mobility shift method, the BPTI domain appears to be correctly folded in all of the fusion proteins in each of the four constructs tested. In the opsin–BPTI fusion, a comparison of the quantity of photolabile pigment produced upon incubation with 11-*cis*-retinal with that detected by protein blots shows that the opsin domain is correctly folded in most of the molecules. Although the experiments reported here did not measure the fraction of each hGH–BPTI fusion containing a correctly folded hGH domain, the ability of each fusion protein to bind to the rGH receptor and biotinylated anhydrotrypsin indicates that in each case some fraction of the molecules contains a correctly folded hGH domain.

The BPTI–trypsin detection system should be useful in a number of applications. As demonstrated here, a hormone–BPTI fusion protein can be used to identify the corresponding receptor produced by transfection of tissue culture cells. This protocol could be adapted to expression cloning of receptors and to the identification of receptors in tissue sections. In the absence of structural information regarding receptor–hormone interactions, these applications would require that a number of different hormone–BPTI fusions be tested for receptor binding activity.

A second application to which the BPTI–trypsin system is well suited is mapping protein topography. The experiments reported here suggest that some surface regions in globular and membrane proteins tolerate BPTI insertions. If, as seems likely, BPTI insertions in the protein interior are not tolerated, then a series of such mutants along the polypeptide chain could be used to define surface-exposed regions. A set of insertion mutants could also be used to map functional topography, for example, those regions involved in protein–protein contact. Functional mapping by BPTI mutagenesis would complement existing high-resolution methods such as alanine-scanning mutagenesis (16) by producing a map in which most mutant proteins could be scored as either active or inactive.

A third application of the BPTI–trypsin system is in protein purification using anhydrotrypsin as an affinity reagent. However, for many proteins the elution conditions required to dissociate the BPTI–anhydrotrypsin complex are likely to result in a loss of biological activity. This problem could be solved by constructing a BPTI derivative with a lower affinity for anhydrotrypsin. Fusion proteins with a lower-affinity BPTI insert could be eluted from anhydrotrypsin by competition with native BPTI.

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